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NUTRITION REVIEWS

VOL. 3 DECEMBER, 1945 No. 12

NUTRITIONAL RESEARCH IN HOLLAND DURING THE WAR

At the end of World War I The Netherlands Institute of Nutrition was founded by Prof. Van Leersum, which, after his death, was removed to the Laboratory of Physiological Chemistry at the University of Amsterdam. At the beginning of this war, nutritional research was stimulated by the foundation of the Section for Nutritional Research of the Dutch Organization for applied research, which in turn founded the Central Institute for nutrition research, under the direction of Dr. Van Eekelen in Utrecht. At the same time, the Board of Nutrition in Holland was founded, a section of which was the Commission for inquiry into the state of nutrition and health of the Dutch people. This section was called the "Pulse Commission" as it held, so to speak, a finger on the pulse of the nation.

In this review is presented a survey of the wartime nutritional research activities of the Amsterdam Institute, many of the activities of which were in cooperation with other laboratories. Much of the work centered around the principal available foodstuffs, potatoes, bread, and dairy products, with particular attention directed toward the supply of thiamine, ascorbic acid, niacin, protein, and the trace elements supplied by these foods.

Potatoes. Contrary to the general belief that potatoes are a source only of carbohydrate, we know now that this vegetable is a food of high quality in other respects. The epidemics of scurvy which in the Middle Ages occurred at the end of the winter in all cities of Western Europe, have disappeared since the introduction of the potato. Furthermore the potato gives a large production per acre. So with the start of the war, many meadows in Holland were transformed into arable land, planted chiefly with potatoes. From 1940 to 1943 the area cultivated with potatoes in Holland was increased from 316,000 to 524,000 acres.

In cooperative projects, our Institute determined the thiamine content of 88 different varieties of potatoes, which varied from 30 to 160 micrograms per 100 g. The deviation amounted to 22 per cent on the average for the same variety grown on one plot and harvested at the same time. For all varieties there was an average of 85 micrograms of thiamine per 100 g. of cooked potato. Approximately 30 per cent of the total thiamine present occurred in the phosphorylated form. These investigations show how largely the potato contributes to the thiamine intake of the Dutch population. Before the war an adult in Holland ate about 500 g. of potatoes per day, and in some months in wartime the consumption was even greater. On an average this furnished 425 micrograms of thiamine, that is, about 30 per cent of the total daily intake.

Potatoes are also the chief source of ascorbic acid in Holland. This was especially true in wartime when no citrus fruits could be imported. Before the war, Holland imported yearly about 100,000,000 kg. of oranges, lemons, and bananas or more than 10 kg. per head of the population.

When potatoes were harvested, their ascorbic acid content was approximately 20 mg. per cent, but during winter storage in pits or huts, the content gradually diminished until in the spring only 5 to 10 mg. per cent was present. Different temperatures of storage, carbon dioxide atmospheres up to 10 per cent, and treatment with the plant hormones, naphthyl-acetic ester and ethylene hydrochloride, did not aid in better retention of ascorbic acid during storage.

The statements in the literature on the

biologic value of potato proteins are conflicting. The growth of young rats on diets that contained 8 and 11 per cent of potato proteins was compared with that of rats on the same diet containing 8 and 11 per cent of casein. Practically no difference was found, hence it was concluded that potato proteins have about the same high biologic value as casein. To go further into this important question, several of the amino acids in potato protein were determined by micromethods. Most of the amino acid contents of potato protein compared favorably with casein, however methionine was a third lower in potato protein and lysine was one-half lower. Based on the figures of Rose for the per cent of each amino acid required in the diet for rat growth, about 30 per cent of the diet should be potato protein (if no other protein were present) to supply the requirement of lysine and methionine. The low methionine content might perhaps be partially compensated for by cystine.

Bread. Quantitatively bread is the chief component of the food in Holland. Before the war 36 per cent of total calories came from bread, and during wartime the proportion remained nearly the same. With the outbreak of the war all imports were stopped, so we had to subsist on our own soil, which is more adapted to the cultivation of rye than of wheat. Therefore the government regulated all the milling and composed a mixture of about two-thirds rye and one-third wheat, to which was added, according to the available stock, small quantities of barley or dried potatoes or potato flour; all bread was prepared from this flour. The question arose as to what extent this "government flour" had to be extracted. A higher extraction gave more flour for bread making, but the digestibility of the bread was lessened, and less bran was left for cattle feeding. An elaborate investigation demonstrated that an extraction of 85 per cent gave the largest output for human consumption, both quantitatively and qualitatively.

A Committee was constituted to determine whether it would be possible to obtain a flour containing a large part of the vitamins and minerals, and yet adapted for baking a more or less white bread of good quality. Members of this Committee included, besides our Institute, the Station for Milling and Baking at Wageningen, the Central Institute for Nutrition Research at Utrecht, the Municipal Institute for the Testing of Food at Amsterdam, and the Dutch Milling Industry.

A large quantity of high grade Dutch wheat was milled for this Committee in the usual way and the different fractions were analyzed by the various laboratories. In our Institute were determined some water soluble vitamins and trace elements. Analyses were made for thiamine, niacin, molybdenum, boron, copper, zinc, manganese, and magnesium in samples of milled wheat ranging from 0 to 31 per cent extraction up to samples of 96 per cent extraction. It was found that the 77 to 79.6 per cent extraction fractions were especially rich in thiamine, and in general contained favorable amounts of the trace elements. Higher extraction fractions contained considerably more niacin. Determinations in other Institutes demonstrated that the 77 to 96 per cent fractions are also rich in enzymes, but that these enzymes greatly diminish the baking quality of the flour. We now plan to investigate whether it will be possible to abolish the harmful influence on the baking quality and retain the special nutritive properties of those fractions. If this proves possible, the nutritive value of the bread would improve considerably.

In conjunction with our interests in the thiamine supply of the diet, studies were made on methods of thiamine determinations of blood. Sinclair has found that 90 per cent of the thiamine of blood is in the phosphorylated form, namely as cocarboxylase. Several methods for measuring thiamine in blood are already known, but either they

require large quantities of blood or they are not accurate. Westenbrink *et al.* have elaborated a new method by adsorbing the cocarboxylase from the blood filtrate by apocarboxylase, which they obtain by washing yeast with a slightly alkaline (pH 8.6) phosphate solution free from cocarboxylase. The adsorption product is carboxylase and its quantity, which depends on the quantity of coferment originally present in the blood, can be determined in a Warburg apparatus by measuring the carbon dioxide liberated from sodium pyruvate. In this way, thiamine can be measured in 2 ml. of blood. By using a Cartesian diver, as devised by Linderstrøm-Lang, the determination could be performed with 0.01 ml. of blood.

Dairy Products. In recent years the nutritive value of margarine was enhanced by adding vitamins A and D. The question then arose whether the nutritive value of this enriched margarine equaled that of butter in all respects. Research in this field seemed desirable, as we had many years ago obtained indications that the particular nutritive value of butter was not based exclusively on its content of vitamins A and D. For this purpose we tested the influence of diet on the growth of young rats. By statistical compilation of our material we could see how narrowly the growth responded to an optimal composition of the diet. We chose a basal diet containing all components necessary for growth except the fats and fat soluble vitamins. To this we added 10 per cent butter, or 10 per cent of other fats supplemented by vitamins A and D. Butter caused a significantly better growth than other (fortified) fats, particularly when summer butter was administered. This growth promoting action was present in the fatty acid fraction of butter, and could be removed by kaolin. Moreover butter was found to contain an antirachitic substance which was not identical with vitamin D_2 or D_3. In studies on processed cheese, it was found that processed cheese has nearly the same vitamin content as natural cheese.

Trace Elements. We know that several trace elements, formerly considered as mere impurities, play an important part in nutrition. The occurrence of endemic goiter in some parts of the world indicates that all trace elements are not always present in optimal quantity in food. Furthermore modern methods of preparing foods remove many of the trace elements.

In our nutrition experiments with refined food mixtures in rats, we always add a mixture of trace elements: molybdenum, copper, manganese, aluminum, zinc, silicon, nickel, cobalt, iodine, fluorine, bromine, boron, arsenic. Research into the importance of trace elements in nutrition and metabolism is simpler than that of vitamins in three respects: (a) their number is limited; (b) for the great majority reliable methods have already been established for their (micro-) determination; and (c) they are stable. However, in another respect their study is more troublesome: their omnipresence. Some fifteen years ago Filedt Kok in our Institute succeeded in lowering the last traces of mineral content of a basic diet somewhat, but it was impossible to remove all traces from the proteins.

We know that in civilized countries dental caries is frequent and it is highly probable that it is caused by inadequate diet. There are in the literature at least two indications that in this case perhaps trace elements might be deficient: ter Meulen found that the molybdenum content of sound human teeth was 0.55 mg. per kilogram, that of carious teeth 0.03 to 0.06 mg. per kilogram. Further, in regions with mottled enamel, caused by excess of fluorine in drinking water, caries seemed to be less frequent. It seemed that caries might be caused by molybdenum or fluorine deficiency. To test this, micromethods were elaborated which could determine the molybdenum and the fluorine content of one separate tooth.

Comparing carious with normal teeth, practically no difference was found in molybdenum or in fluorine content. This problem is continuing in another direction, by determining the content of trace elements of crude and prepared foods. Toward this end, micromethods for the determination of copper, manganese, zinc, boron, magnesium, and aluminum have been developed.

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ALCOHOL AND THE THIAMINE REQUIREMENT

The study of Jolliffe (*Quart. J. Studies on Alcohol* **1,** *74 (1940)*) which correlated clinical polyneuritis with dietary history, indicated that the caloric intake from alcohol was involved in production of the thiamine deficiency. In the experiments of Strauss (*Am. J. Med. Sci.* **189,** *378 (1935)*), clinical improvement of polyneuritis occurred upon feeding a well balanced diet with yeast and liver extract although the patients were allowed to continue their usual intake of alcohol. Since it was well established that the thiamine requirement is increased by a high carbohydrate intake, the thesis seemed to follow that thiamine is particularly required in the metabolism of alcohol as well as of carbohydrate. This belief seemed to receive substantiation from another direction with the report of Westerfeld, Stotz, and Berg (*J. Biol. Chem.* **144,** *657 (1942)*) that pyruvate accelerated alcohol metabolism, apparently by a mechanism which involved an enzyme containing thiamine. Further studies by these same workers (*Ibid.* **149,** *237 (1943)*) revealed that the primary effect of pyruvate was due to a coupled oxidation-reduction which did not require thiamine directly but that metabolism of acetaldehyde, the first product of alcohol oxidation, definitely required thiamine (*Ibid.* **152,** *41 (1944)*). These studies seem to prove that alcohol metabolism requires thiamine but do not indicate the relative need of carbohydrate and alcohol for thiamine.

There is now evidence that alcohol may actually decrease the thiamine requirement. Lowry, Sebrell, Daft, and Ashburn (*J. Nutrition* **24,** *73 (1942)*) showed that polyneuropathy and death in rats on a thiamine deficient diet were delayed by ingestion of alcohol, whether this replaced the carbohydrate or was in addition to the usual diet. These experiments not only do not support the earlier contention, but indicate that alcohol may even decrease the thiamine requirement of carbohydrate metabolism.

The dependence of carbohydrate metabolism on thiamine is evident in the accumulation of pyruvate during thiamine deficiency. If alcohol metabolism were likewise critically dependent on thiamine, it would be expected that the rate of alcohol removal would be adversely affected during thiamine deficiency. However, Berg, Stotz, and Westerfeld (*J. Biol. Chem.* **152,** *51 (1944)*) could not find any decrease in the rate of alcohol metabolism or on the effect of pyruvate on alcohol removal even during acute phases of thiamine deficiency in pigeons.

Westerfeld and Doisy (*J. Nutrition* **30,** *127 (1945)*) have further investigated the question of the role of thiamine in alcohol metabolism. They have chosen as an experimental object the pigeon, in which thiamine deficiency has been extensively studied and forced feeding of chosen diets can be readily instituted. Four groups of 12 birds each were given isocaloric diets (thiamine deficient) containing the same amounts of casein, autoclaved yeast, cod liver oil, salt mixture, choline, alpha-tocopherol, and 2-methyl-1,4-napthoquinone. The variations consisted of changes in the corn oil, sucrose, and alcohol contents of the diets. Diet I

was high in carbohydrate, in Diet II part of the carbohydrate was replaced by alcohol, and in Diet III partly by fat. Diet IV had part of the fat of Diet III replaced by alcohol. Each diet was force-fed daily in an amount equivalent to approximately 100 calories. Death from acute thiamine deficiency occurred in the different groups within the following average periods: on Diet I, nineteen days; Diet II, twenty-five days; Diet III, twenty-seven days; and Diet IV, forty-two days. Both fat and alcohol showed a thiamine sparing action, 16 g. of fat being approximately equal to 9 g. alcohol in this regard.

In a second series of experiments, these investigators first produced the opisthotonos stage of deficiency in a group of pigeons, then gave a single curative dose of 25 micrograms and placed groups of birds on the four diets previously described. A daily dose of 10 micrograms of thiamine was given for a period of two weeks, and then withdrawn for a successive observation period. The length of the period required to cause the reappearance of opisthotonos was considered a measure of the relative thiamine requirements of the diets. These experiments were designed to eliminate some of the variables of the first series, but the results showed qualitatively the same results.

It was concluded that the isocaloric substitution of alcohol for dietary fat or carbohydrate delays the onset of thiamine deficiency. It would appear that alcohol requires less thiamine for metabolism than carbohydrate. The investigators also offer the possible explanation that the metabolism of alcohol and carbohydrate together requires less thiamine than that of carbohydrate alone. An attempt was made to study the thiamine requirement on a diet free of carbohydrate, with or without alcohol, but the pigeons died at irregular periods and generally before any signs of thiamine deficiency occurred.

These latest experiments lend little support to the belief that alcohol requires thiamine for its metabolism. In the clinical etiology of polyneuropathy in alcoholism, it would appear that the thiamine intake must be even less than the lowered requirement resulting from the replacement of carbohydrate by alcohol.

INFLUENCE OF pH ON GROWTH FACTOR REQUIREMENTS OF FUNGI AND BACTERIA

Observations have been made concerning the effect of pH on the growth factor requirements of fungi and bacteria which are of interest both to students of this phase of nutrition and to individuals who are employing microbiologic technics for assay purposes. It has been shown that variations in the pH of the medium profoundly influence the required concentrations of various factors for maximum growth of different organisms. It has been demonstrated, further, that in some instances the dispensability of certain growth factors is determined by pH.

Stokes, Foster, and Woodward (*Arch. Biochem.* **2**, *235* (*1943*)) observed that a "pyridoxineless" mutant of Neurospora sitophila could grow normally without pyridoxine under sharply defined conditions of pH and nitrogen nutrition. Without pyridoxine, the fungus did not develop in the pH range 4.9 through 5.4 but gave maximum growth in the range 6.2 to 7.3 inclusive. The ability to grow without pyridoxine was exhibited even in the favorable pH range only when ammonium nitrogen was supplied. With other forms of nitrogen the vitamin always was required. Synthesis of pyridoxine when the fungus grew without the vitamin was demonstrated by assay of extracts with Neurospora mutant at pH

4.9 and with Lactobacillus casei. Stokes *et al.* made the further interesting observation that the mutant was stimulated by thiamine when grown at pH 4 to 5 with submaximum levels of pyridoxine.

Brueckner (*Yale J. Biol. Med.* **15,** *813 (1943)*) noted that nicotinic acid was ten times more efficient in promoting the growth of Staphylococcus aureus at pH 6.2 than at pH 7.6. Nicotinamide was twice as efficient at the lower pH.

Doede (*Yale J. Biol. Med.* **17,** *595 (1945)*) made a further investigation of these phenomena. She studied nicotinic acid (and nicotinamide) as a growth factor for S. aureus and Shigella paradysenteriae Sonne at pH 6.0, 7.0, and 8.0, and "folic acid," riboflavin, pyridoxine, pantothenic acid, biotin, and nicotinic acid as growth factors for L. casei at pH 5.0, 6.0, and 7.0.

Some twenty-four strains of S. aureus were tested on Gladstone's medium. It was found that the minimum concentration of nicotinic acid which would support maximum growth in five days at a pH of 8.0 averaged for the various strains twelve times that required at a pH of 6.0. More nicotinamide also was needed at the higher than at the lower pH but the difference was not as great as for nicotinic acid. These findings are in essential agreement with those of Brueckner.

When Gladstone's medium (consisting of a mixture of fifteen amino acids, salts, and glucose) was used with S. paradysenteriae Sonne, Doede found that nicotinic acid was not required at pH 6.0 although it was required at pH 7.0 or 8.0. This held true for all of the four strains tested. Using a simpler medium containing inorganic salts, asparagine, and glycerol, nicotinic acid was required at all pH levels but sixteen times as much was needed for maximum growth at pH 8.0 as at pH 6.0. Further tests showed that this organism could easily be trained to grow without nicotinic acid at all three pH levels even on the simpler medium. It was shown in addition that the organism so trained synthesized its own nicotinic acid (or, as stated by the author, a substance capable of replacing nicotinic acid for L. casei or for untrained S. paradysenteriae Sonne). When an ammonium phosphate was substituted for asparagine in the medium, it was not found possible to train the organism to grow without nicotinic acid.

L. casei was grown on a basic medium of amino acids, salts, and glucose to which was added riboflavin, "folic acid," nicotinic acid, biotin, pyridoxine, and pantothenic acid. Acid production during sixty-six to seventy-two hours of incubation following inoculation was used as the measure of growth. A pH of 7.0 proved to be unfavorable for this organism and growth was submaximal. "Folic acid," riboflavin, biotin, and pantothenic acid were more effective at pH 6.0 than at pH 5.0. Nicotinic acid was equally effective at the two pH levels while pyridoxine was found to be more effective at pH 5.0. In addition, it was found that at pH 5.0 considerable growth occurred without pyridoxine, about one-fourth as much acid being produced as with the optimal amount of the vitamin. No growth occurred without pyridoxine at pH 6.0 or 7.0.

From the foregoing it is evident that the growth requirements of organisms may be affected quantitatively and in some instances may even be changed qualitatively, by changes in the pH of the medium. It appears, therefore, that for the sake of accuracy in microbiologic assays, mediums should be adequately buffered. For an adequate explanation of the effects exerted by pH a more detailed knowledge of bacterial metabolism than we at present possess will be necessary.

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